The cell surface receptor G6b, a member of the immunoglobulin superfamily, binds heparin

Edwin C.J.M. de Vet, Stephen A.B. Newland, Paul A. Lyons, Begoña Aguado[1], R. Duncan Campbell*

MRC Rosalind Franklin Centre for Genomics Research, Hinxton, Cambridge CB10 1SB, United Kingdom

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Abstract **The *G6b* gene, located in the human Major Histocompatibility Complex, encodes a receptor of the immunoglobulin (Ig) superfamily. In this study, we show using a variety of techniques that the extracellular domain of the G6b protein, containing a single Ig-like domain, binds to heparin with high affinity. In an ELISA assay, this binding was displaceable with soluble heparin with an IC50 value of approximately 0.5 μg/ml. Other sulfated glycans showed weaker or no competition. The observed interaction between G6b and heparin is strongly salt dependent suggesting a mainly electrostatic interaction. Heparin might modulate the interaction of G6b with its as yet unidentified protein ligand.**

Keywords: Immunoglobulin superfamily; Heparin; Sulfated glycan; MHC

1. Introduction

The *G6b* gene, located in the class III region of the Major Histocompatibility Complex, encodes a novel cell surface receptor of the immunoglobulin (Ig) superfamily [1]. This superfamily contains a large group of cell surface receptors involved in cellular recognition and signal transduction [2]. The *G6b* gene undergoes alternative splicing giving rise to multiple membrane bound and soluble isoforms. The two major membrane bound isoforms (G6b-A and G6b-B) contain a single Ig-like domain and transmembrane segment, but differ in their cytoplasmic tails. G6b-B contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic tail which are not found in the G6b-A isoform. G6b-B binds the protein tyrosine phosphatases SHP-1 and SHP-2 after pervanadate-induced phosphorylation of these ITIMs, classifying this isoform as a new member of the family of inhibitory receptors [1].

A crucial part in understanding the in vivo function of the G6b protein is the identification of its extracellular ligand. Protein ligands for Ig superfamily members are often, but not exclusively, other Ig superfamily members [2]. Interactions between Ig superfamily members and their respective ligands are usually low affinity which makes the identification of those ligands experimentally challenging [3,4]. However, certain Ig superfamily members have been reported to bind to non-protein molecules such as the siglec family of receptors which bind sialic acid [5]. In addition, other cell surface proteins, such as members of the fibroblast growth factor receptors and selectins [6], as well as a number of cytokines including interleukin-2 [7], interleukin-6 [8] and interleukin-12 [9], have been shown to bind to heparin and heparan sulfate, highly sulfated, negatively charged, glycosaminoglycans that are widely distributed in the extracellular matrix and on the cell surface. The binding of proteins to heparin is thought to play an essential biological role. For example, in the case of the basic fibroblast growth factor receptor heparin/heparan sulfate is essential for this protein to bind to its ligand [10,11]. In the case of cytokines it has been suggested that heparin-like glycans retain the secreted cytokine close to their sites of secretion [9] thus giving rise to localized high concentrations of the cytokine.

In this study, we show using a variety of techniques that the extracellular domain of the G6b protein binds to heparin and provide evidence that this interaction is mainly electrostatic. Although a protein ligand for the extracellular part of G6b has not been identified, the binding of heparin might play an essential role in the biological function of G6b.

2. Materials and methods

2.1. Cloning of expression constructs

The constructs encoding full length N-terminally tagged G6b-A and G6b-B are described elsewhere [1]. As negative controls full length human CD86 (NM_175862) and human SIRPalpha (BC033092) were cloned under the control of the CD33 leader peptide and containing an N-terminal T7 epitope tag similar to the cloning of N-terminally tagged G6b-A and G6b-B [1]. To create a soluble protein the cDNA encoding the extracellular domain of G6b including the endogenous hydrophobic signal peptide (amino acids 1–142) was amplified by PCR using a full length clone as template. The forward primer created a *Hin*dIII site (underlined) TTATAAGCTTACCATGGCTGTGTTTCTGC and the reverse primer introduced a *Not*I site (underlined) ATCCGCGGCCGCCTGGGGATACACGGACCC. This PCR fragment was cloned into the *Hin*dIII–*Not*I sites of the pIgplus vector (Novagen) creating a construct encoding the leader peptide and

*Corresponding author. Fax: +44 1223 494512.
E-mail address: rcampbel@rfcgr.mrc.ac.uk (R.D. Campbell).

[1] Present address: Centro Nacional de Biotecnología, Campus Cantoblanco, Madrid, Spain.

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FGF, fibroblast growth factor; Ig, immunoglobulin; PBS, phosphate buffered saline; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis

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extracellular part of G6b fused in-frame with the Fc-tail of human IgG1 (G6b-Fc). The extracellular domains of the human G6f protein and human CD152 were cloned as Fc fusions in a similar way.

2.2. Heparin agarose binding assay

Proteins were transiently expressed in Cos-7 cells using the DEAE dextran method in 6 well plates as described before [12]. For pull down assays with the full length cellular proteins the transfected Cos-7 cells were washed with PBS and lysed in lysis buffer (10 mM Tris/HCl pH 7.5, 1% NP-40, 150 mM NaCl, 0.02% sodium azide) supplemented with protease inhibitor cocktail (Sigma) and 1 mg/ml BSA (0.6 ml/well). An aliquot of the lysate was mixed with approximately 10 μl of heparin-agarose gel (Sigma) in 200 μl of lysis buffer containing 1 mg/ml BSA in the absence or presence of 1 mg/ml soluble heparin. The mixture was rotated at room temperature for 1 h and the beads were spun down and washed twice with lysis buffer. Proteins were eluted from the beads by addition of Laemmli SDS–PAGE sample buffer [13] containing 5% β-mercaptoethanol. Samples were directly analysed by Western blot immunostaining and proteins were detected with the anti-T7 tag mAb (Novagen) as described before [1]. Pull down assays with the soluble Fc-fusion proteins were performed by mixing 100 μl of culture supernatant containing the soluble recombinant protein with 10 μl heparin agarose gel in 100 μl lysis buffer supplemented with 0.1 mg/ml BSA. The mixture was incubated for 1 h at room temperature. Beads were spun down and washed twice with lysis buffer. Proteins were eluted from the beads with Laemmli SDS–PAGE sample buffer and analysed by Western blot immunostaining. The Fc-fusion proteins were detected with a goat anti-human IgG (γ-chain specific) peroxidase-conjugated antibody (Sigma).

2.3. FACS analysis of transfected HEK293 cells

HEK293 cells were grown in DMEM medium supplemented with 10% foetal calf serum and penicillin/streptomycin and were transfected in 6 well plates (2.5×10^5 cells/well) with Effectene (Qiagen) according to the manufacturer's protocol. The constructs encoding full length G6b-A and G6f (N-terminally tagged with the T7 epitope) have been described before [1,14]. Cells were harvested three days after transfection by resuspending them in fresh medium. For the detection of heparin binding, 2.5×10^5 cells per incubation were spun down, washed in PBS and incubated for 45 min at room temperature in PBS containing 0.2 mg/ml BSA and 50 μg/ml heparin-biotin. Cells were spun down, washed with PBS/BSA and incubated with streptavidin-PE-Cy5 (BD Pharmingen) for 45 min at room temperature. Cells were spun down and fixed in PBS/1% formaldehyde. To detect expression of the T7-tagged proteins on the cell surface, cells were also stained with the T7 tag mAb using anti-mouse IgG-FITC as secondary antibody. FACS analysis was done on a Beckman Coulter EPICS-XL machine.

2.4. Heparin ELISA

The Fc-fusion proteins were expressed in Cos-7 cells for three days and then purified batchwise from culture supernatants using protein A-Sepharose (Sigma). Typically, to 30 ml of culture supernatant 50 μl of protein A-Sepharose gel was added and the mixture was incubated for 4 h at 4 °C under rotation. The protein A-Sepharose gel was washed twice with cold PBS and the Fc-fusion proteins were eluted with 0.1 M glycine pH 3.2. The eluates were neutralized with Tris to pH ~ 7.5 and BSA was added to a final concentration of 0.1 mg/ml. Proteins were stored in aliquots at −20 °C.

A 96 well flat bottom ELISA plate (Greiner) was coated overnight with 5 μg/ml avidin in PBS (100 μl/well) at 4 °C. The rest of the experiment was done at room temperature. The plate was washed with PBS and wells were incubated with heparin-biotin (Sigma) in PBS (20 μg/ml, 100 μl/well) for 2 h. After washing with PBS the wells were blocked with 2% milk powder in PBS for 1 h. The plate was washed with PBS/0.05% Tween-20 and wells were incubated for 2 h with the Fc fusion proteins (approximately 12 ng/well) in 80 μl PBS/0.2 mg/ml BSA in the absence or presence of various glycan/sugars which were purchased from Sigma. In the case of the measurement of the influence of NaCl on the binding of G6b-Fc to heparin, 10 mM Tris/HCl (pH 7.5) was used instead of PBS with NaCl added at the indicated concentrations. The wells were washed three times with PBS/Tween and incubated for 30 min with a 1:1000 dilution of goat anti-human (Fc specific) alkaline phosphatase conjugate (Sigma) in PBS/2% milk powder. Wells were washed three times with PBS/Tween and subsequently three times with PBS. Alkaline phosphatase was detected by adding 100 μl of *p*-nitrophenyl phosphate substrate (Sigma) to the wells. Reactions were stopped typically after 10–15 min with EDTA and the OD405 was measured using a Fusion™ α plate reader (Packard Bioscience). Wells not incubated with a Fc fusion protein were used as blanks.

3. Results

3.1. Heparin agarose binding assay

To establish a direct interaction between heparin and G6b a heparin-agarose pull down experiment was performed. Both solubilised T7-tagged full length G6b-A and G6b-B interacted with heparin-agarose whereas other T7-tagged proteins bound to a much lesser extent (CD86) or not at all (SIRPalpha) under identical experimental conditions (Fig. 1, left panel). A fusion protein containing only the extracellular part of G6b fused to the Fc tail of human IgG1 (G6b-Fc) also interacted with heparin-agarose whereas other Fc fusions (G6f and CD152) or the Fc domain itself did not (Fig. 1, right panel). These interactions could be efficiently blocked by the addition of soluble heparin (1 mg/ml) indicating that the extracellular part of G6b recognizes heparin on the beads (Fig. 1), an interaction that might be biologically relevant.

3.2. Binding of heparin to HEK293 cells

HEK293 cells, which do not express endogenous G6b, were transfected with either G6b-A or empty vector (pcDNA3). Fig. 2 shows that cells transfected with G6b can bind heparin whereas cells transfected with the empty vector do not bind heparin. Staining with the T7 mAb showed that the cells were efficiently transfected with G6b (Fig. 2, right panel). Furthermore, HEK293 cells transfected with full length G6f and

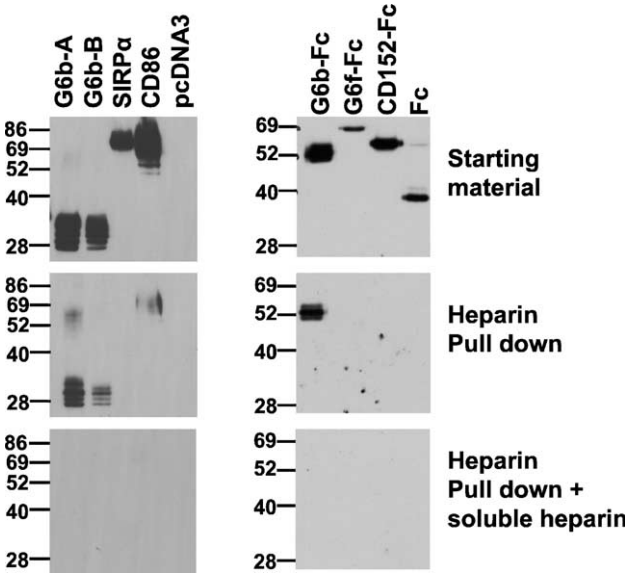

Fig. 1. Western blot analysis of heparin-agarose binding assay. A heparin-agarose binding assay was performed on full length T7-tagged solubilised proteins (left panels) and soluble Fc fusion proteins (right panels). The starting material for both experiments is shown in the upper panels, the eluates from heparin-agarose in the middle panels, and the eluates from heparin-agarose when the experiment was performed in the presence of soluble heparin (1 mg/ml) in the lower panels. The Western blots are representative of three identical experiments.

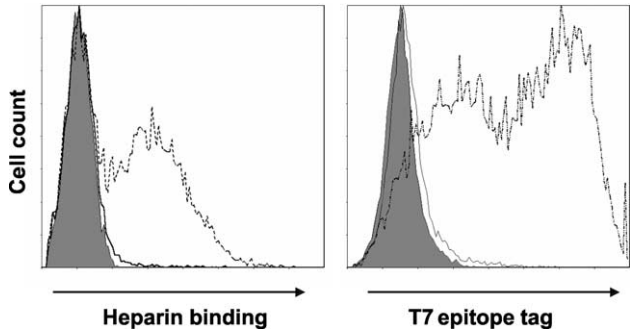

Fig. 2. FACS analysis of binding of heparin-biotin to G6b expressed on the surface of HEK293 cells. Cells were transfected with empty vector (pcDNA3) (closed line) or T7-tagged full length G6b-A (hashed line) and after 3 days were stained with heparin-biotin (left panel) or the T7 mAb (right panel). Unstained and untransfected cells are shown in grey.

SIRPalpha did not bind heparin (data not shown). This experiment shows that the extracellular domain of G6b expressed on a cell surface is able to bind heparin.

3.3. Heparin ELISA

To investigate whether other glycans were able to compete with the binding of G6b to heparin, an ELISA based approach was used. As shown in Fig. 3A, the G6b-Fc fusion protein showed binding to immobilized heparin in this assay whereas G6f-Fc, CD152-Fc and Fc alone did not bind. These experiments were carried out in PBS, at physiological salt (NaCl) concentration (0.15 M). Subsequently, this assay was used to determine whether various glycans could inhibit the binding of G6b-Fc to immobilized heparin. Soluble heparin was the most efficient competitor to binding of G6b-Fc to immobilized heparin with an IC50 value of the order of 0.5 μg/ml (Fig. 3B). Certain sulfated glycans like fucoidan, heparan sulfate and chondroitin sulfate B were also able to compete although not as efficiently as heparin yielding much higher IC50 values of approximately 3, 100 and 10 μg/ml, respectively. Sialic acid, hyaluronic acid, chondroitin sulfate C and sodium sulfate did not compete with the binding of G6b-Fc to immobilized heparin. We also observed that the addition of EDTA at a concentration of 5 mM did not affect the binding of G6b-Fc to immobilized heparin making the involvement of a divalent cation in the interaction unlikely (data not shown). Furthermore, the heparin disaccharide I-S (Sigma) did not inhibit binding of G6b-Fc to heparin at concentrations as high as 100 μg/ml (data not shown) which suggests that heparin binds to G6b through more than two sugar residues.

To investigate whether the interaction between heparin and G6b was governed by electrostatic interactions, we examined the effect of NaCl concentration on the binding of G6b to heparin in the ELISA. Although the G6b-Fc fusion protein clearly binds to immobilized heparin with high affinity at physiological salt concentration as shown by the data in Fig. 3, about 3-fold more binding is observed in the presence of 100 mM NaCl (Fig. 4). As the binding of G6b-Fc to immobilized heparin is strongly salt dependent this suggests a strong electrostatic contribution to the binding of G6b to heparin.

4. Discussion

In this paper, we show that the extracellular domain of the cell surface molecule G6b binds heparin. In an ELISA-based approach, it was found that soluble heparin competed for the binding of G6b to immobilized heparin with an IC50 value of 0.5 μg/ml. This value is of the same order of magnitude as those obtained for the binding of heparin to interleukin-12 (0.1 μg/ml) [9], FGF-2 (0.15 μg/ml) and anti-thrombin III (1 μg/ml) [7]. This indicates an affinity of G6b for heparin comparable to these high-affinity heparin-binding proteins. Other sulfated glycans were able to compete for the binding of G6b to heparin although with much lower affinities. This suggests a specificity of G6b for heparin. However, highly sulfated fucoidan (based on fucose residues) is a much more efficient competitor than heparan sulfate which is structurally more related to heparin than fucoidan. For that reason it is possible that it is the level of negative charge/sulfation on the glycan that determines the observed high affinity of G6b for heparin

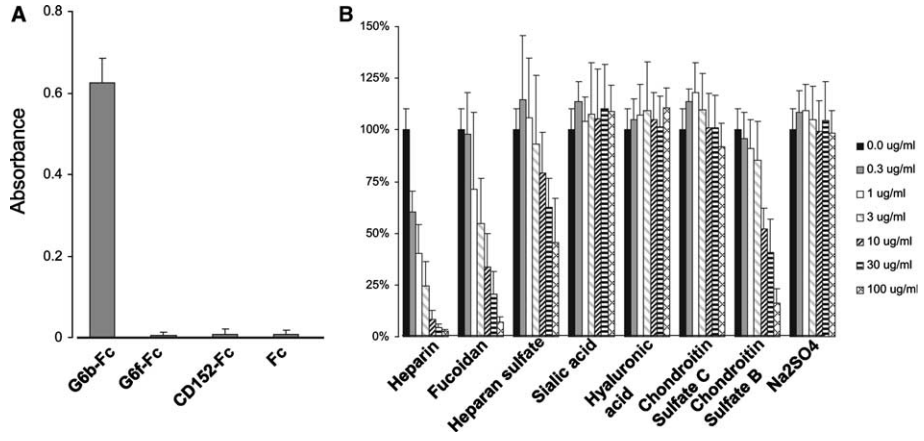

Fig. 3. ELISA of G6b-Fc binding to immobilized heparin. (A) Binding of various Fc-fusion proteins to immobilized heparin. (B) Effect of various glycans/sugars on the binding of the G6b-Fc protein to immobilized heparin. The ELISA experiment was performed in the presence of the indicated sugar/glycans as well as Na_2SO_4 at the indicated concentrations. Results are expressed as the percentage of signal observed with G6b-Fc in the absence of any potential inhibitor. Results are averaged over six independently performed experiments.

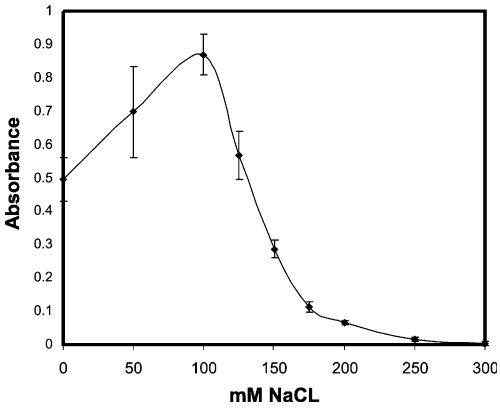

Fig. 4. Salt dependence of the binding of the G6b-Fc protein to immobilized heparin. The binding of G6b-Fc to heparin was performed at the indicated NaCl concentrations.

rather than a distinct structural feature of this molecule. The strong influence of the NaCl concentration on this interaction lends further support to the notion that the interaction is mainly electrostatic.

Stretches of positively charged residues in the primary sequence have been identified as heparin binding motifs, for example the C-terminal proximal clusters of 4–5 basic residues that give rise to the heparin binding properties of IFN-γ [15] and interleukin-8 [16]. However, basic residues that are far apart in the primary sequence can be close in the folded protein. Some heparin binding proteins are known to contain large positively charged surfaces which mediate high affinity binding of heparin [17]. In this respect it should be noted that the extracellular domain of G6b is particularly enriched in positively charged residues, especially arginines (12 arginines in a 125 amino acid domain) which are known to mediate strong binding to heparin [18]. The Ig fold is well characterised (see [19] for a detailed description of the Ig fold) and it can be hypothesized that in the G6b protein a number of basic residues (R43, K54, K58, R60, R61, K109, R111 and R117) will be located in that part of its Ig-like domain made up of the GFCC' β-sheet strands. These residues might form a positively charged surface able to bind heparin. In addition, a number of basic residues are likely to be located in that part of the Ig-like domain of G6b made up of the ABED β-sheet strands (R26, R30, R83, R85, R92 and R93) and these could also be involved in heparin binding.

The physiological relevance of the observed interaction between G6b and heparin remains to be established. The protein ligand of G6b is unknown hampering further investigations. It is a possibility that heparin binding modulates the interaction between G6b and its as yet unidentified protein ligand as is observed with the interaction of FGF with various FGF receptors [11]. Alternatively, G6b might mediate the adhesion of cells to heparin in the extracellular matrix or present on the surface of other cells. Once a protein ligand for G6b is identified, a more detailed study of the functional importance of the observed interaction between heparin and G6b can be undertaken.

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